EVIDENCE FOR A DISTINCT GROUP OF NESTIN-IMMUNOREACTIVE NEURONS WITHIN THE BASAL FOREBRAIN OF ADULT RATS

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Abstract—Nestin is an intermediate filament protein serving as a marker for neuroprogenitor and stem cells. Here we report that a cluster of previously unrecognized nestin immunoreactive (nestin-ir) neurons was located in the medial septum–diagonal band of Broca (MS-DBB) of the basal forebrain in adult rats. Nestin-ir neurons were exclusively located in the MS-DBB and intermingled with choline acetyltransferase-ir (ChAT-ir), parvalbumin-ir (PV-ir), or nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase reactive (NADPHd-reactive) neurons. However, there was no colocalization between nestin-ir and PV-ir in single neurons in MS-DBB; only about 35% of nestin-ir neurons were ChAT-ir, and 8%–12% of nestin-ir neurons were NADPHd-reactive. Morphologically, nestin-ir neurons showed a larger size of somata than that of ChAT-ir or PV-ir neurons and the distribution of nestin-ir neurons spread across the rostro-caudal extent of the MS-DBB. Moreover, retrograde tracing revealed that a significant portion of these nestin-ir neurons projected to the thalamus and hippocampus. These results, for the first time, provide strong evidence that there exists a cluster of previously unrecognized nestin-ir neurons in MS-DBB of the basal forebrain in adult rats and that these nestin-ir neurons are distinguishable from ChAT-ir, PV-ir, and NADPHd-reactive neurons. © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: nestin, cholinergic, GABAergic, colocalization, immunohistochemistry, basal forebrain.

The basal forebrain is a heterogeneous region with multiple types of neurons and various brain connections (Chandler and Crutcher, 1983; Freund and Antal, 1988; Gaykema et al., 1991; Jakab and Léránth, 1990; Semba, 2000). Neurons in the basal forebrain contain acetylcholine, GABA, nitric oxide synthase, and various neuropsin...
Nestin has been recently discovered as an intermediate filament component protein expressed by immediate precursor cells of neuron and glia during the brain development (Lendahl et al., 1990). It has been extensively used as a marker for CNS progenitor cells because its in vivo expression correlates with many CNS regions with proliferating progenitor cells (Dahlstrand et al., 1995), although cells expressing nestin may not be exclusively progenitor cells (Gage, 2000). In this study, we identified a distinct group of nestin-ir neurons in the rostral part of the basal forebrain (MS-DBB) in adult rats, which may be the third population of neurons in this area previously suggested by Smith and Booze (1995).

**EXPERIMENTAL PROCEDURES**

Sprague-Dawley rats (S.D., Charles River Laboratory, Wilmington, MA, USA) of various ages were used. The experimental protocols were approved by our Institutional Animal Care and Use Committee and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize the number of animals used and their suffering. Animals were housed under controlled temperature (21 °C ± 2 °C), relative humidity (50% ± 10%) and artificial lighting (12-h light/dark cycle, lights on at 7 A.M.). All animals had ad libitum access to distilled water and a standard rat diet. The nomenclature from the rat's brain atlas by Paxinos and Watson (1997) was used to describe the basal forebrain and nuclei such as MS, VDB and HDB in that region.

**Western blot**

Western blot was used to confirm the specificity of mouse anti-rat nestin monoclonal antibody (Rat-401) and also to quantify the expression of nestin in postnatal rats. Samples from the basal forebrain of 18 sibling rats (n=3 per time point) were obtained at postnatal days (P) 1, 3, 7, 14, 28, and 56. Separate samples of the basal forebrain were harvested from additional rats of 12 and 16 weeks as well as 6 and 12 months of age (n=3 per time point). Rats were anesthetized using sodium pentobarbital (60 mg/kg body weight, intraperitoneally, i.p.) before being decapitated. Brain samples were removed and immediately placed on dry ice and stored at −80 °C until use. Samples were homogenized in SDS sample buffer containing a mixture of proteinase inhibitors (Sigma, St. Louis, MO, USA). Protein samples were separated on SDS-PAGE gels (4–7.5% gradient gel) and transferred to polyvinylidene difluoride filters (Millipore, Bedford, MA, USA). The filters were blocked with 5% milk for one hour at room temperature and incubated overnight at 4 °C with mouse anti-rat nestin antibody (Rat-401, Pharmingen, San Diego, CA, USA, 1:1000), followed by HRP-conjugated secondary antibody (1:7000; Amersham Biosciences, Arlington Heights, IL, USA) incubated for one hour at room temperature. The blots were visualized in ECL solution (NEN, Boston, MA, USA) for one minute and exposed onto hyperfilms (Amersham Biosciences) for 1–10 min. The blots were then incubated in a stripping buffer (67.5 mM Tris, pH 6.8, 2% SDS, and 0.7% β-mercaptoethanol) for 30 min at 50 °C and reprobed with mouse anti-β-actin antibody (1:20,000; Abcam Inc., Cambridge, MA, USA) as a loading control. All Western analysis was made in triplicates. The Western blot band density was measured with Photoshop and normalized against corresponding loading controls. Differences were compared using SPSS by repeated measure one-way ANOVA.

**Histochemistry and immunostaining**

Fifteen male rats of three months old were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused transcardially with cold phosphate-buffered saline (PBS, 0.01 M, pH 7.35) followed by 4% paraformaldehyde in phosphate buffer (PB, 0.1 M, pH 7.35). The forebrain (AP-2–AP -4 ) blocks were harvested on a cryostat (Leica, Bensheim, Germany, 30 μm). Coronal sections from AP -4 to AP -1 were listed into a 24-cell board, hole-by-hole and round-by-round, and floated in PBS. Eight sets (sets A–H) of sections were included and processed for histochemistry or immunostaining.

Set A was used to detect colocalization between nestin (Rat-401, mouse monoclonal, 1:100; Pharmingen) and NSE (neuron specific enolase, rabbit anti-rat polyclonal, 1:100; Chemicon, Temecula, CA, USA), NeuN (neuronal nuclei, mouse anti-rat monoclonal, 1:2000; Chemicon) or GFAP (gliarial fibrillary acidic protein, rabbit anti-rat polyclonal, 1:1000; Chemicon). Sets B, C and D were processed for immunostaining for nestin, ChAT (rabbit anti-rat polyclonal, 1:2000; Chemicon) or PV (rabbit anti-rat polyclonal, 1:2000; Chemicon) alone. Sets E and F were used for double-immunostain of nestin and ChAT or PV, respectively. Set G was used for double staining of NADPHd histochemistry or neuronal nitric oxide synthase (nNOS) (rabbit anti-rat polyclonal, 1:250; BD Biosciences, San Jose, CA, USA) and nestin immunohistochemistry. Set H was used as a negative control by incubating the sections in normal mouse serum (1:1000; Becton Dickinson, Franklin Lakes, NJ, USA) instead of a primary antibody. A free-floating method was used for all staining processes including immunofluorescence and indirect peroxidase for immunohistochemistry. Antibodies were diluted in PBS (pH 7.4) containing 1% bovine serum albumin and 0.3% Triton X-100. PBS was used in all rinsing steps and the staining was carried out at room temperature unless indicated otherwise.

For immunofluorescence staining, sections were washed (3×10 min), blocked with 3% donkey serum in 0.1 M PBS containing 0.3% Triton for 1 h at room temperature, and incubated overnight at 4 °C with a primary antibody. After rinsing (3×10 min), sections were incubated for 1 h at room temperature with a corresponding FITC- or Cy3-conjugated secondary antibody (donkey polyclonal, 1:800; Jackson ImmunoResearch, West Grove, PA, USA). Sections for nestin and GFAP-ir were also counterstained with DAPI (Invitrogen, Carlsbad, CA, USA) to show the lack of co-localization between nestin and GFAP. Sections were subsequently washed and mounted onto chrome alum-coated slides, coverslipped with Vectashield (Vector Laboratories, Burlingame, CA, USA). For double staining, a second primary antibody was added after the completion of staining for the first primary antibody.

For indirect peroxidase immunostaining, sections were washed (3×10 min) followed by a 30-min incubation in 1% hydrogen peroxide in PBS to clear endogenous peroxidases. After rinsing (4×5 min), 3% normal goat serum was applied for 30 min to prevent non-specific conjugate binding. Sections were incubated with a primary antibody for 2 h at 37 °C and then for 14 h at 4 °C. After rinsing (3×5 min), corresponding biotin-conjugated IgG (goat anti-mouse or goat anti-rabbit, 1:200; Jackson Immunoresearch) was added and incubated for 120 min. Following rinsing (3×5 min), avidin–biotin peroxidase complex (ABC kit, Vector, 1:200) was added and again incubated for 120 min. The peroxidase reaction was visualized by diaminobenzidine (DAB, Sigma, 0.05%, containing 0.01% H2O2 in PBS) for 3–8 min. For double labeling, sections were stained first by a primary antibody and visualized by DAB. After again incubated in 0.3% H2O2 for 30 min and then 5%
normal goat serum for 30 min, the sections were incubated for 2 h at 37 °C and 14 h at 4 °C with a second primary antibody.

The second peroxidase reaction was visualized with the 3,3'-5,5'-tetramethylbenzidine (TMB)—ST method. Thus, after re-action with a second primary antibody and incubated with ABC complex, the sections were rinsed in 0.01 M PBS (3×5 min), again in distilled water (2×10 min), and incubated (15 min in dark) in 50 ml TMB-ST solution (Solution A: sodium tungstate 1 g, distilled water 47 ml, 0.2 M PBS of pH 5.0–5.4 with 1 N HCl 1.5 ml; Solution B: TMB 7 mg, acetone 0.5 ml, anhydrous alcohol 1 ml; mixing solution A and B upon use), H2O2 (0.3%; 7 μl/ml) was then added for several times (no more than six times) separated by intervals of 10 min under the microscopic view until the second primary antibody reactive cells turned into clear green. The section was again rinsed in 0.05 M PBS (pH 5.0–5.4; 3×5 min). After the final rinsing, sections were mounted, air-dried, dehydrated and coverslipped with neutral balsam.

For double labeling of NADPHd and nestin, sections were rinsed in PBS first and then incubated in 0.2 M PBS (pH 8.0) containing nitroblue tetrazolium (0.5 M, Sigma) and β-NADPHd (1 mM, Sigma) at 37 °C for 0.5–1 h until the sections were satisfactorily developed under the microscopic view. The sections were washed in 100 mM sulfuric acid to stop the reaction. After rinsing (3×5 min), the sections were processed for nestin immunostaining as described above.

For the data processing, sections were visualized using a microscope (Olympus America Inc., Center Valley, PA, USA), recorded using a digital camera, and processed using Adobe Photoshop (Adobe Systems, San Jose, CA, USA). Five representative coronal sections (from brain section of AP1.2–AP1.6 L2 H5.9: H refers to the depth in mm from the skull surface) were examined from every rat. The number of nestin-, ChAT-, and PV-ir cells was counted using a microscope (Olympus). The statistical software SigmaPlot 2000 was used for data analysis.

RESULTS

Age-dependent expression of nestin in the basal forebrain

In this experiment, the mouse anti-rat nestin monoclonal antibody (Rat-401) was pivotal for all immunostaining procedures and the validity of its specificity to nestin protein was confirmed by Western blot as shown by a single band located at 200 kDa consistent with the size of nestin (Fig. 1). Nestin was abundantly expressed in the basal forebrain during the early postnatal period (P1, P3 and P7) but downregulated quickly afterward. By P14, nestin was no longer detectable when the same amount of sample (50 μg) as that for P1, P3, and P7 was loaded (Fig. 1). However, when the loading sample was doubled to 100 μg, nestin was detectable up to P56. When the loading sample was further increased to 200 μg, nestin was also detectable at time points of 12 and 16 weeks as well as six months. By the age of 12 months, however, nestin could not be detected even with the sample load of 200 μg, although the positive loading control (actin) remained detectable at all times. The results indicate that there was an age-dependent progressive downregulation of the nestin expression in rats.

Nestin-ir cells expressed both NSE and NeuN

Immunostaining results showed that nestin-ir cells existed in the basal forebrain of adult rats (Figs. 2–7). To identify the type of nestin-ir cells, double immunofluorescent labeling was performed to examine the possible colocalization between nestin and NSE or NeuN (neuronal markers) as well as nestin and GFAP (a glial marker) in adult rats (3 months old). In double-labeled sections, nestin co-expressed with NSE and NeuN but not with GFAP (Fig. 2). Since NeuN has been shown to be absent in progenitor cells but present in mature neurons (Schwartz et al., 2003; Gage, 2000; Gage et al., 1995), the results indicate that...
these nestin-ir cells were mature neurons instead of progenitors or glial cells.

Distribution patterns of nestin-ir neurons in the MS-DBB in relation to ChAT- or PV-ir neurons

Nestin-ir neurons existed exclusively in the MS-DBB including the MS, VDB and HDB regions (Fig. 3a, b). No nestin-ir neurons were found in the ventral globus pallidus, substantia innominata and lateral preoptic region. In the caudal sections of the HDB, which was equivalent to the basal nuclear level, only few nestin-ir neurons were observed. In total, nearly 85% of detected nestin-ir neurons were located in the rostral extent of MS-DBB (bregma AP 0.2–AP 0.7). As compared with nestin-ir neurons, ChAT-ir or PV-ir positive neurons were distributed much more extensively across the basal forebrain. The distribution of ChAT-ir neurons was consistent with that reported in the literature (Fibiger, 1982; Houser et al., 1983; Schwaber et al., 1987; Kiss et al., 1990a,b; Woolf, 1991). These ChAT-ir neurons were located in the MS, VDB, HDB, ventral pallidum and substantia innominata. In the MS-DBB complex, nestin-ir neurons intermingled with ChAT-ir neurons (Fig. 4). Moreover, in the caudal part of the basal forebrain, only few nestin-ir neurons were detected, which scattered among extensively distributed ChAT-ir neurons in the same region without evidence of co-localization. Overall, the distribution of nestin-ir neurons was similar to that of ChAT-ir neurons in the Ch1–Ch3 but not Ch4 groups.

The distribution of PV-ir neurons was generally in agreement with that described in earlier studies (Celio, 1986; Heizmann and Celio, 1987; Kiss et al., 1990a,b). PV-ir positive neurons were distributed extensively in the basal forebrain including such regions as the MS-DBB, ventral globus pallidus, substantia innominata, and the lateral preoptic region. In the anterior part of the MS, the distribution of nestin-ir neurons was similar to that of PV-ir neurons. However, nestin-ir neurons were seen more laterally in the posterior part of the MS and VDB, while PV-ir neurons were located closer to the middle line. In the HDB, the majority of nestin-ir neurons intermingled with PV-ir neu-
rons while the distribution of nestin-ir neurons was closer to the ventral part and the PV-ir neurons closer to the dorsal part (Fig. 5a, d). Overall, the ratio of nestin-ir neurons to PV-ir neurons in the MS-DBB was approximately 1:2.

**Morphology of nestin-ir neurons in comparison with ChAT-ir and PV-ir neurons in the MS-DBB**

Nestin-ir (DAB method) was consistently seen as fine yellowish-brown reaction products that filled both cytoplasm and proximal dendrites of nestin-ir neurons within the basal forebrain. The dendritic labeling extended to a considerably long distance from the soma, and the proximal axons were also occasionally stained. Under a high magnification microscopic view, many varicosity-like structures were seen along nestin-ir dendrites while NeuN were not stained, leaving round ‘vacuum’ holes of various sizes (Fig. 3c, d).

The morphologic profile of nestin-ir neurons appeared to be similar to that of ChAT-ir or PV-ir neurons within the MS-DBB. ChAT-ir and PV-ir neuronal somata were smaller than those of nestin-ir neurons, ranging from a medium (8–12×20–25 μm) to large (10–15×25–35 μm) size. All nestin-ir, ChAT-ir and PV-ir somata were generally smaller...
in the medial septal nuclei than in the diagonal band. All three types of neurons varied in their morphology from fusiform to multipolar (Figs. 3–7). Nestin-ir neurons often sent off one or two long dendrites, which ran dorsoventrally within the MS (Fig. 3a) and followed the curved ventral surface of the hemisphere in the diagonal band (Fig. 3b, c; Figs. 4 and 5). These results indicate that nestin-ir neurons were unlikely to be local interneurons but projection neurons from the basal forebrain.

Colocalization between nestin-ir and ChAT-ir, PV-ir, or NADPHd-reactive neurons

In those sections double-stained with nestin and ChAT or nestin and PV visualized with DAB and TMB, nestin-ir neurons were seen as yellowish-brown whereas ChAT-ir or PV-ir neurons as light green (Fig. 4a; Fig. 5a). ChAT-ir or PV-ir neurons were lightly stained as compared with the strongly stained nestin-ir somata against the lightly stained nuclei. As such, different types of neurons were readily distinguishable from each other due to color differences. There were fewer nestin-ir neurons than ChAT-ir or PV-ir neurons in the basal forebrain, but the number of neurons among these three groups was similar in the rostral extent of the MS-DBB (Table 1). Colocalization between nestin and ChAT could be seen in the same section, such that about 35% of nestin-ir neurons were ChAT immunopositive whereas 25% ChAT-ir neurons were nestin immunopositive (Fig. 4d). However, no colocalization was observed between nestin-ir and PV-ir in any single neuron under the examination (Fig. 5a–d).

After a sequential staining for NADPHd histochemistry followed by nestin immunohistochemistry, an increase in the DAB reaction product was observed. NADPHd reactivity (in blue) could be clearly distinguished from the yellowish-brown nestin-ir product. NADPHd-reactive neurons were similar in morphology and distribution to nestin-ir neurons in the MS-DBB (Fig. 6a). Although the majority of nestin-ir neurons within the MS-DBB intermingled with NADPHd-reactive neurons (about 1:1 ratio with regard to total neurons in each category), only a few (about 8%) nestin and NADPHd double-stained (blue–brown) neurons were observed. This result was confirmed in immunostained sections in which colocalization between nestin-ir and nNOS-ir were present in about 12% of nestin-ir neurons (Fig. 6d).

Taken together with the double-labeling data between nestin-ir and ChAT-ir, PV-ir or nNOS-ir neurons, these results indicate that most nestin-ir neurons formed a group of neurons distinguishable from those ‘traditional’ ChAT-ir, PV-ir, and NADPHd-reactive neurons in the basal forebrain.
Retrograde tracing of nestin-ir neurons

The retrograde tracer (Fast Blue) reached the basal forebrain at four days after its injection into the thalamus or hippocampus. There was a mix of Fast Blue–labeled, nestin-ir, and Fast Blue/nestin-ir double-labeled neurons in the MS-DBB (Fig. 7; Table 2). Fast Blue injected into the hippocampus more often reached the MS and VDB, whereas Fast Blue injected into the thalamus more often reached the HDB, indicating possible differential projections from the basal forebrain. The majority of Fast Blue/nestin-ir double-labeled neurons were located in the MS-DBB ipsilateral to the injection, only a few double-labeled neurons were observed on the contralateral side of this region, suggesting that nestin-ir neurons mainly sent projections ipsilaterally with only scattered contralateral or bilateral projections. In contrast, Fast Blue injected into the frontal cortex and anterior olfactory nucleus was nearly absent in nestin-ir neurons in the basal forebrain, suggesting a lack of projection from nestin-ir neurons in the basal forebrain to these brain regions.

DISCUSSION

The present study demonstrated that 1) a previously unidentified population of nestin-ir neurons existed in the MS-DBB of adult rats; 2) although the distribution pattern of these nestin-ir neurons was similar to that of ChAT-ir, PV-ir, and NADPHd-reactive neurons, there was no colocalization between nestin-ir and PV-ir neurons and only minimal colocalization (8%–12%) with NADPHd-reactive neurons, as compared with more extensive colocalization (25%–35%) between nestin-ir and ChAT-ir neurons; 3) nestin-ir neurons exhibited a different morphological profile including a larger size of their somata as compared with that of ChAT-ir and PV-ir neurons; and 4) nestin-ir neurons in the MS-DBB projected mainly to the ipsilateral hippocampus and thalamus. These results, for the first time, provide strong evidence for the existence of a distinguishable population of nestin-ir neurons within the basal forebrain of adult rats.

As a member of intermediate filament protein, nestin was once considered as a special marker for neuroprogenitor or stem cells (Lendahl et al., 1990; Dahlstrand et al., 1995). Although nestin is abundantly and transiently expressed in multipotential stem cells during the embryogenesis (Lendahl et al., 1990) and in proliferating neuronal cells in the developing CNS (Frederiksen and McKay, 1988), nestin is quickly downregulated in the CNS in postnatal animals and replaced by other types of intermediate filaments at the end of differentiation (Cattaneo and McKay, 1990; Liem, 1993), except for vascular epithelium and ependymal cells (Lois and Alvarez-Buylla, 1994; Li...
and Chopp, 1999). In adult rats, the nestin expression has been detected only in reactive astroglial cells following CNS trauma or ischemia (Li and Chopp, 1999; Duggal et al., 1997; Morshead et al., 1994; Shibuya et al., 2002) and in sustentacular cells in the mature olfactory neuroepithelium (Doyle et al., 2001). Of note is that although the nestin gene is also quickly downregulated after postmitotic differentiation, nestin-ir neural stem cells do exist in adult rats (Reitze et al., 2001; Johansson et al., 1999; Reynolds and Weiss, 1992; Richards et al., 1992; McKay, 1997; Gage, 2000; Doetsch et al., 1999). Such cells are located in the forebrain ependyma, subventricular zone (Coskun and Luskin, 2002; Luskin, 1993; Luskin et al., 1997; Johansson et al., 1999; Doetsch et al., 1999; Chiasson et al., 1999; Gritti et al., 1999) and the rostral migratory stream (Luskin, 1993). Nestin-ir cells have also been reported in the adult human basal forebrain (Gu et al., 2002). In the present study, we identified a population of nestin-ir neurons in adult rats without any CNS injury, which existed exclusively in the MS-DBB and were colocalized with both NSE and NeuN (two distinct neuronal markers). These nestin-ir neurons are apparently mature neurons that are different from neuroprogenitor or stem cells as discussed above, because these cells were positively stained with NeuN (Schwartz et al., 2003) and sent projections primarily to the hippocampus and thalamus.

The basal forebrain sub-regions are heterogeneous with respect to the neurotransmitter phenotype. Retrograde tracers injected into the hippocampus and cortex have shown that approximately 35–50% of the medial septal neurons projecting to the hippocampus are cholinergic neurons (Rye et al., 1984). At least some non-cholinergic projections to the hippocampus arising from the MS have been identified as GABAergic neurons (Linke et al., 1994). Besides cholinergic and GABAergic neurons, there are various peptidergic neurons in the basal forebrain including those containing galanin, somatostatin, neuropeptide Y, neurotensin, or substance P (Freund, 1989; Hiatt et al., 1992; King and Anthony, 1984; Kiss et al., 1990b; Kivipelto and Panula, 1986; Köhler and Eriksen, 1984; Krnjevic et al., 1988; Kitchener and Diamond, 1993; Senut et al., 1989). Out data indicate that there also exists a group of nestin-ir neurons in the MS-DBB of adult rats. Of significance is that although the distribution pattern of nestin-ir neurons was similar to that of ChAT-ir, PV-ir, nNOS-ir and NADPHd-reactive neurons within the MS-DBB, the double-labeling results showed no colocalization between nestin-ir and PV-ir neurons. Only 8%–12% of

Fig. 6. Distribution of nestin-ir and NADPHd-reactive or nNOS-ir neurons in the MS-DBB. Double-stained sections with nestin-ir and NADPHd histochemistry or nNOS-ir showing the relationship between nestin-ir (brown in a, green in b and d) and NADPHd-reactive (blue in a) or nNOS-ir (red in c and d) neurons. (a) Part of the MS and VDB, showing colocalization between nestin-ir and NADPHd-reactivity in some neurons (blue-brown, arrow); scale bar=62.5 μm. (d) Merged from b and c, showing colocalization between nestin-ir and nNOS-ir in some neurons (yellow, arrow); scale bar=100 μm.
nestin-ir neurons were colocalized with NADPHd-reactive or nNOS-ir neurons and about 25%–35% of nestin-ir neurons were also ChAT positive. This limited colocalization (or lack colocalization in the case of PV) between nestin-ir and ChAT-ir, PV-ir, or NADPHd-reactive/nNOS-ir neurons strongly indicates that nestin-ir neurons in the MS-DBB of the basal forebrain in adult rats are primarily non-cholinergic, non-GABAergic, and non-nitrergic neurons, given that (1) ChAT-ir and NADPHd-reactivity/nNOS-ir are regarded as specific markers for cholinergic and nitrergic neurons, respectively (Hahn et al., 1992; Holscher 1997) and (2) the vast majority (>90%) of the PV-ir neurons are also GAD-ir (Gritti et al., 2003) thereby GABAergic neurons (Freund, 1989; Kiss et al., 1990a) in the MS-DBB. The exact chemical nature of this distinct group of nestin-ir neurons in the MS-DBB of the basal forebrain in adult rats remains to be determined, considering that most neuropeptides-containing neurons co-localize with either cholinergic or GABAergic neurons (Semba, 2000; Pasqualotto and Vincent, 1991).

The MS-DBB of the forebrain connects mainly with the hippocampus and cerebral cortex. Neurons in this region are a major source of cholinergic innervation to the cortex, hippocampus, amygdala and olfactory bulb (Rye et al., 1984). In the present study using the retrograde tracing method, we found that at least some of these nestin-ir neurons projected to the hippocampus, suggesting a distinct septohippocampal pathway in addition to the known cholinergic and GABAergic projections. If this is the case, nestin may be used as a marker to identify this non-cholinergic and non-GABAergic septohippocampal path-

<table>
<thead>
<tr>
<th>Area</th>
<th>Neuron type</th>
<th>Nestin-ir</th>
<th>ChAT-ir</th>
<th>PV-ir</th>
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<tr>
<td>MS</td>
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<td>62.7±9.5</td>
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<td>VDB</td>
<td>44.8±8.3</td>
<td>52.8±8.1</td>
<td>76.7±20.2</td>
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<tr>
<td>HDB</td>
<td>117±27.7</td>
<td>116±21</td>
<td>85.3±46.4</td>
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way. Our present findings may also have functional implications. It has been reported that neurons in the basal forebrain are essential to the process of learning, memory and cognition (Semba, 2000; Bartus et al., 1982). Nestin-ir cells have been reported in the adult human basal forebrain and these cells tend to decline in the process of aging (Gu et al., 2002). Therefore, this distinct group of nestin-ir, non-cholinergic, non-GABAergic, and non-nitricergic neurons in the MS-DBB of the adult rat’s basal forebrain may be an important target for studying the mechanisms of neurodegenerative and cognitive disorders including Alzheimer’s disease.

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REFERENCES


Table 2. Fast Blue–labeled neurons in the ipsilateral basal forebrain

<table>
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<tr>
<th>Area</th>
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<th>Thalamus</th>
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<td>Fast Blue</td>
<td>Double-labeled</td>
<td>Percent of nestin-ir*</td>
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<tr>
<td></td>
<td>Fast Blue</td>
<td>Double-labeled</td>
<td>Percent of nestin-ir*</td>
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<tr>
<td>MS</td>
<td>27.4 ± 7.4</td>
<td>34.3 ± 11.4</td>
<td>7.3 ± 3.2</td>
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<tr>
<td>VDB</td>
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<tr>
<td>HDB</td>
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<td>47.1 ± 21</td>
<td>9.7 ± 3.1</td>
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<td>21.5 ± 2.1</td>
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<td>21 ± 8.3</td>
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<td>25.8 ± 12.9</td>
<td>7.4 ± 3.4</td>
<td>12.7%</td>
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*Percent of nestin-ir neurons retrogradely labeled by Fast Blue; data are expressed as mean ± S.E.M.